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NON-GENETIC BASED PROTEIN DISEASE MARKERS

FIELD OF THE INVENTION

The present invention relates the discovery of protein markers for diseases that do not have a genetic component.

BACKGROUND OF THE INVENTION

The studies of identical twins have allowed one to study non-genetic factors without concern for polymorphisms and mutations as the twins originated from the same zygote and thus are genetically identical. While post separation genetic mutations may occur, these are relatively few compared to the large number of differences between fraternal twins or unrelated individuals.

When examining many proteins by two dimensional electrophoresis gels from monozygotic twins and unrelated individuals, the twins always gave identical protein displays for PHA stimulated lymphocytes, Goldman et al, American Journal of Human Genetics 35(5):827-37 (1983). The same results occurred when detecting polymorphisms in serum proteins, Borresen et al, Clinical Genetics 20(6):438-48 (1981). Using two dimensional electrophoresis gels to determine protein differences for Huntington's disease also resulted in no characteristic protein on the gel.

Monozygotic twins were shown to correlate for serum carboxyl terminal propeptide of type I procollagen, PICP serum pyridinoline crosslinked carboxyterminal telopeptide of type I collagen, ICTP, and serum aminoterminal propeptide of type III

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procollagen. Tokita, et al, Journal of Clinical Endocrinology & Metabolism 78(6):1461-1466 (1994).

Monozygotic twins correlated with each other for various serum proteins in twin pairs that developed diabetes versus those twin pairs that did not. Hussain, et al, Diabetologia 39 (1):60-69 (1996)and Roder, et al, Journal of Clinical Endocrinology and Metabolism 80(8):2359-63 (1995). Likewise for hypertension McCaffery, et al, Journal of Hypertension 17 (12):1677-85 (Dec. 1999)(not prior art) and Robertson et al, American Journal of the Medical Sciences 318(5):298-303 (1999) (not prior art).

Selby, et al, American Journal of Epidemiology 125 (6):979-88 (1987) argue environmental factors as important to diabetes as they correlate to whether monozygotic twins have or not have diabetes. Likewise, for Kesaniemi, et al, Acta Genet Med Gemellol (Roma) 33(3): 467-73 (1984).

Correlation between monozygotic twins was noted for obesity as opposed to dizygotic twins. Selby, et al, Journal of the American Medical Association 265(16):2079-2084 (1991).

Correlation for monozygotic twins for obesity has been noted by Lemieux, International Journal of Obesity 21(10):831-838 (1997); Obesity in Europe 91. Proceedings of the 3rd European Congress on Obesity (edited by Ailhaud, et al, Vol. 062 Abs. No. 04070 John Libbey & Company Ltd. London, UK; Pritchard et al, Metabolism 48(9):1120-7 (1999)(not prior art); Narkiewicz, et al, Journal of Hypertension 17(1):27-31 (1999); Pritchard, et al, Journal of Clinical Endocrinology and Metabolism 83(9):3277-84 (1989); Hong, et al, Arterioscler. Thromb. Vasc. Biol. 17(11):2776-82 (1997) and Oppert, et al, Metabolism 44(1):96-105 (1995).

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Monozygotic twins who are phenotypically discordant for schizophrenia have been proteometricly examined by two-dimensional gel electrophoresis, Vander Putten, et al, Biol. Psychiatry 40(6):437-442 (1996). The authors report that one protein was significantly elevated between an affected twin and its control twin and that the same protein was significantly elevated when unrelated schizophrenic patients were compared to unrelated normal control individuals.

CARMELLI, HEART, LUNG, AND BLOOD INSTITUTE AWARD TYPE-Noncompeting Continuation (Type 5) FISCAL YEAR- 1998 to SRI INTERNATIONAL examined monozygotic twins over 23 years of follow-up examining obesity, essential hypertension and non-insulin-dependent diabetes mellitus (NIDDM) for discordant presence.

When comparing monozygotic twins, genetics did not appear to have any effect on obesity and neither appears to account for the variation in hormone values in twin pairs. Meikle, et al, Metabolism 37(6):514-7 (1988). Serum lipids, lipoproteins, and lipid metabolizing enzymes in identical twins discordant for obesity were compared.

Monozygotic twins discordant for obesity have been examined regarding certain serum lipoproteins. Ronnemaa, et al, Journal of Clinical Endocrinology and Metabolism 83(8):2792-9 (1998), Hayakawa et al, Atherosclerosis 66(1-2):1-9 (1987) and for plasma leptin concentrations, Ronnemaa, et al Annals of Internal Medicine 126(1):26-31 (1997) and Ronnemaa et al, Journal of Clinical Endocrinology and Metabolism 85(8):2728-32 (2000)(not prior art).

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SUMMARY OF THE INVENTION

The object of the present invention is to discover and use protein markers for a disease state and the markers per se.

It is a further object of the present invention to determine protein markers that result from the disease state and which do not appear because of normal genetic variation.

It is another object of the present invention to provide diagnostic markers for obesity, diabetes, osteoporosis osteoarthritis and hypertension and to diagnose and stratify these and other diseases by measuring the amount of one or more markers in a biological sample.

It is still a further object of the present invention to determine the degree of severity of a disease state, its prognosis, the preferred choice of therapy and/or efficiency of therapy by measuring the relative amounts of each of the disease markers and ratios between each and/or other conventional measures of the disease state.

It is yet another object of the present invention to provide suitable targets for drug discovery of compounds that are agonists or antagonists of a protein and to screen candidate compounds with such targets.

It is another object of the present invention to compare the relative efficacy of candidate pharmaceuticals and diagnostics by comparing the relative effect on one or more disease marker between candidates or between a candidate and an established pharmaceutical or diagnostic.

It is still another object of the present invention to determine coregulating proteins that may be used to determine at least parts of a metabolic pathway.

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It is another further object of the present invention to find methods for regulating a first protein by affecting a second protein.

It is yet another further object of the present invention to determine efficacy of a treatment for a disease by measuring these protein markers during or after treatment and comparing to a positive control, a negative control or the individual before treatment.

It is a still another object of the present invention to determine sets of presumably related proteins which when taken together constitute a protein marker.

Other aspects of the invention include the protein markers themselves, proteomic displays containing abnormal abundances of the protein markers, and their many uses for research and monitoring patients. Also, combinations of plural proteins constituting a combination marker and submarkers co-fluctuating with other markers may be used as other protein markers.

The present invention accomplishes this goal by determining which proteins are present in abnormal abundances in biological samples and optionally deducing the mechanism of action from the perturbed metabolic pathway. Initially, all readily detectable proteins are measured; but after the markers are determined, an assay for the markers alone is sufficient. In addition, monitoring of either patients on the drug, laboratory animals in drug discovery or pre-clinical and clinical testing protocols may utilize such an assay. Sets of perturbed protein markers provide a proteomic pattern or "signature" for better determination also indicating aspects of and the status of the diseased state.

The present invention determined non-genetic disease protein markers by searching for proteins present in abnormal

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abundances between monozygotic twins where the twins are discordant for the disease state. Initially, all readily detectable proteins are measured in a biological sample to determine which are disease markers; but after the markers are determined, an assay for the markers alone is sufficient for diagnosis. In addition, monitoring of either patients on the drug or laboratory animals in drug discovery or pre-clinical testing protocols for efficacy may utilize such an assay.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is an image of a two dimensional electrophesis gel of human serum master pattern of HUSERFRAC3A-1 with hits highlighted in red and the MSN spot number.

Figure 2 is an image of a two dimensional electrophesis gel of human serum master pattern of HUSERFRAC3A-1 POPULATIONS with groups of hits highlighted in red and the MSN spot number for the individuals and the groups given.

Figure 3 is an image of a two dimensional electrophesis gel of human serum master pattern of HUSERFRAC3A-2 with hits highlighted in red and the MSN spot number.

Figure 4 is an image of a two dimensional electrophesis gel of human serum master pattern of HUSERFRAC3A-2 POPULATIONS with groups of hits highlighted in red and the MSN spot number for the individuals and the groups given.

Figure 5 is an image of a two dimensional electrophesis gel of human serum master pattern of HUSERFRAC5-1 with hits highlighted in red and the MSN spot number.

Figure 6 is an image of a two dimensional electrophesis gel of human serum master pattern of HUSERFRAC5-1 POPULATIONS with groups of hits highlighted in red and the MSN spot number for the individuals and the groups given.

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Figure 7 is an image of a two dimensional electrophesis gel of human serum master pattern of HUSERFRAC5-2 with hits highlighted in red and the MSN spot number.

Figure 8 is an image of a two dimensional electrophesis gel of human serum master pattern of HUSERFRAC5-2 POPULATIONS with groups of hits highlighted in red and the MSN spot number for the individuals and the groups given.

Figure 9 is an image of a two dimensional electrophesis gel of human serum master pattern of HUSERFRAC6-2 with Hits highlighted in red and the MSN spot number.

Figure 10 is an image of a two dimensional electrophesis gel of human serum master pattern of HUSERFRAC5 Alpha group with the ALPHA 1 AT group of hits highlighted in red and the MSN spot number for the individuals and the groups given

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The term "diabetes" in this application refers to Diabetes Mellitus, Type II or non-insulin-dependent diabetes (NIDDM) or insulin resistance.

The term "isolated", when referring to a protein, means a chemical composition that is essentially free of other cellular components, particularly most other proteins. The term "purified" refers to a state where the relative concentration of a protein is significantly higher than a composition where the protein is not purified. Purity and homogeneity are typically determined using analytical techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. Generally, a purified or isolated protein will comprise more than 80% of all macromolecular species present in the preparation. Preferably, the protein is purified to greater than 90% of all macromolecular species present. More

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preferably, the protein is purified to greater than 95% and most preferably, the protein is purified to essential homogeneity, or wherein other macromolecular species are not significantly detected by conventional techniques.

The term "protein" is intended to also encompass derivatized molecules such as glycoproteins and lipoproteins as well as lower molecular weight polypeptides.

The term "protein marker" is a detectable "protein" which has its concentration, abundance, derivatization status, activity or other level altered in a statistically significant way when a host producing the protein marker has a diseased state. Many protein markers are disease specific and all denote an amount property and use of the "protein".

A "level" refers to abundance, derivatization status, protein variant presence, concentration, chemical or biological activity, which is detectable. An "altered level" refers to a change in the "level" when compared to a different sample. The "level" may be an actual measured amount of a protein but is generally a relative "level" of a protein compared to the "level" of other proteins or standards, preferably several hundred from the same gel.

"Small molecules" are low molecular weight preferably organic molecules that are recognizable by receptors. Typically, small molecules are specific binding components for proteins.

The terms "binding component", "ligand" or "receptor" may be any of a large number of different molecules, and the terms are used interchangeably sometimes.

The term "ligands" refers to chemical components in a sample that will specifically bind to receptors. A ligand is typically a protein or peptide but may include small molecules,

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particularly those acting as a hapten. For example, when detecting proteins in a sample by immunoassay, the proteins are the ligands.

The term "receptors" refers to chemical components in a reagent, which have an affinity for and are capable of binding to ligands. A receptor is typically a protein or peptide but may include small molecules. For example, an antibody molecule acts as a receptor.

The term "bind" includes any physical attachment or close association, which may be permanent or temporary. Generally, an interaction of hydrogen bonding, hydrophobic forces, van der Waals forces, etc., facilitates physical attachment between the ligand molecule of interest and the receptor. The "binding" interaction may be brief as in the situation where binding causes a chemical reaction to occur. This is typical when the binding component is an enzyme and the analyte is a substrate for the enzyme. Reactions resulting from contact between the binding component and the analyte are within the definition of binding for the purposes of the present invention. Binding is preferably specific. The binding may be reversible, particularly under different conditions.

The term "bound to" or "associated with" refers to a tight coupling of the two components mentioned. The nature of the binding may be chemical coupling through a linker moiety, physical binding or packaging such as in a macromolecular complex. Likewise, all of the components of a cell are "associated with" or "bound to" the cell.

"Labels" include a large number of directly or indirectly detectable substances bound to another compound and are known per se in the immunoassay and hybridization assay fields. Examples include radioactive, fluorescent, enzyme,

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chemiluminescent, hapten, spin labels, a solid phase, particles, etc. Labels include indirect labels, which are detectable in the presence of another added reagent, such as a receptor bound to a biotin label and added avidin or streptavidin, labeled or subsequently labeled with labeled biotin simultaneously or later.

In situations where a chemical label is not used in an assay, alternative methods may be used such as agglutination or precipitation of the ligand/receptor complex, detecting molecular weight changes between complexed and uncomplexed ligands and receptors, optical changes to a surface (e.g., in the Biacore® device) and other changes in properties between bound and unbound ligands or receptors.

An "array" or "microarray" (depending on size) is generally a solid phase containing a plurality of different ligands or receptors immobilized thereto at predetermined locations. By contacting ligands under binding conditions to the microarray, one can determine ligand or receptor identity or at least part of the ligands' structure based on its location on the microarray. While not a single solid phase, a series of many different solid phases (or other labeling structure) each with a unique receptor immobilized thereon is considered a microarray. Each solid phase has unique detectable differences allowing one to determine the ligand or receptor immobilized thereon. An array may contain different receptors in physically separate locations even when they are not bound to a solid phase, for example a multi-welled plate.

The term "disease-related marker or portions thereof" as used herein refers to particular compounds or complexes which are found in abnormal abundances in a disease.

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The term "disease state" refers to the disease condition or extent of the condition that an individual possess. It includes the prognostic situation and other details of the individual's disease as well which can be used for a variety of indications.

The term "biological sample" includes tissues, fluids, solids (preferably suspendable), extracts and fractions that contain proteins. These protein samples are from cells or fluids originating from an organism. The biological sample may be taken directly from the organism or tissue being affected or indirectly from the organism such as from body fluids such as blood, plasma, serum or urine. In the present invention, the host is generally an animal, preferably a human when the diseases are obesity, osteoporosis, diabetes, osteoarthritis or hypertension, and may be any type of organism when discussing disease in general.

The term "proteome" is a large number of proteins expressed in a biological sample, representing the total, relevant portion or preferably all detectable proteins by a particular technique or combination of techniques. "Proteome analysis" is generally the simultaneous measurement of at least 100 proteins, generally at least a few hundred proteins, preferably over 1000 and most preferably plural thousands of detectable proteins from a sample when separated by various techniques. In the present invention, the proteome analysis involves two-dimensional gel electrophoresis. While this is the generally accepted technique for analyzing proteomes, other techniques are acceptable and may be used for the present invention if they generate large numbers of quantitatively detectable proteins. Another example is discussed in U.S. Patent Application Serial Number 60/166,266.

The term "target" refers to any protein perturbed by a disease, developmental stage or after drug treatment.

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Frequently, a target refers to a drug development target that is capable of binding, or being altered by, an agent. Such drug development targets are suitable for screening candidate compounds either using direct binding assays or by observing a perturbed level, thereby indicating the candidate compound is appropriate for the next level of drug screening.

The terms "host", "subject", "individual", and "sample of interest" include normal or abnormal organisms, and various tissues, cells and fractions (including subcellular fractions) of each of these.

Monozygotic twins that are discordant for a disease trait represent a good system for studying diseases as the cause cannot be related to any genetic process. Furthermore, monozygotic twins are so identical that almost any difference in their proteins is important. In the present invention, biological samples are taken from each twin and the quantity and quality of every protein in the biological sample's proteome is compared. When the twins differ dramatically with respect to a particular disease state, the perturbations in the proteome are likely to be caused by the disease state or at least such perturbations represent identifiable markers associated with the disease state.

In the preferred embodiment, the biological samples from many discordant identical twins are subjected to proteometric analysis whereby the quantity of every protein in a twin's sample is compared to its respective partner (if any) in the respective twin sample. The data is analyzed statistically by conventional methods for determining a correlation between each perturbed protein and disease state. The results, given in the tables below are lists of significant markers for each respective disease state.

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In many biological samples, a few common proteins constitute a large percentage by weight or mole of all proteins present by weight or mole. These common proteins are typically the least interesting from a disease standpoint and the most interfering. Such common proteins generate large spots and smears on a two-dimensional electrophoresis gel thereby interfering with the measurement of other proteins. To complicate matters, there are limits to how much protein can be run on a two-dimensional electrophoresis gel before the gel becomes fragile and physically breaks into many pieces. Since some of the proteins in the biological samples occur in low concentrations, they may be present below the detectable limit of the system.

To reduce this interference, the common uninteresting proteins are first removed from the protein sample before it is loaded into the electrophoresis separation system. This also and enhances sensitivity as the protein sample being loaded is depleted of unwanted proteins allowing a higher amount of low abundance proteins to be loaded into the system producing a relatively higher amount of such low abundance proteins so that they can be detected.

In the present invention, this was done by immunosubtraction and/or sample fractionation but a variety of other fractionation methods may be used. By reacting the naturally common proteins to an immobilized specific binding agent such as an antibody, they are effectively and selectively removed from the sample. Furthermore, fractionating the sample mixture into various fractions based on various physical properties, such as the presence or absence of glycosylation, still larger amounts of low abundance proteins may be loaded into the electrophoresis system. Any of a long list of

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conventional protein separation and purification techniques, well known per se, may be used.

In the preferred embodiment, commercially available solid phase beads (Poros®) having Protein G or Protein A bound thereto and mixtures of them are first contacted with an antibody recognizing one of the proteins. As protein A and protein G bind to the constant region of the antibody molecule, it does not interfere with antibody-antigen binding, thereby maintaining optimal orientation for their affinity towards the serum proteins.

The antibodies are then cross-linked to the Protein G or A after binding by the dimethylpimelimidate method to form stable amide linkages. Schneider et al, Journal of Biological Chemistry 257:10766-10769 (1982). Because interaction between amine groups was minimized due to previous binding of antibody to Protein G or A, the affinity binding sites were minimally affected.

In the present invention, a correlation having a probability value of $p<0.01$ was accepted as indicating statistical significance. While higher p values of <0.01 may be considered statistically acceptable to some, markers of greater statistical significance may be more desirable for diagnostic, prognostic, indicative of which therapy to use and monitoring the effects of therapy to ameliorate the disease state.

Since all p value cut-offs represent a somewhat arbitrary threshold, it is possible and likely to miss significant protein markers using one embodiment of the present invention. However, by looking at related diseases or artificial disease models, which may be related by mechanism of action, one can find proteins with altered abundance with respect to the controls. Even though not statistically significant alone, if such a

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protein were found to be altered in biological samples from for example an animal model, the result can be considered statistically significant. When determining what is to be considered a protein marker, a protein may constitute a disease marker even when not statistically significant in a single experiment with one causative agent alone.

Total protein markers identified by direct correlation or ANOVA are listed below.

The protein markers, which are perturbed by various disease states, are as follows. When different variants of the proteins are present and used as markers, references to the different MSN numbers is given.

Table 1: Non-Genetic Markers for Obesity

MSN Number/Master
19 / HUSERFR3A
316 / HUSERFR3A
420 / HUSERFR3A
501 / HUSERFR3A
680 / HUSERFR3A
1139 / HUSERFR3A
S 2 / HUSERFR3A
S 22 / HUSERFR3A
S 24 / HUSERFR3A
S 33 / HUSERFR3A
519 / HUSERFR6
39 / HUSERFRAC5
72 / HUSERFRAC5
96 / HUSERFRAC5
101 / HUSERFRAC5

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104 / HUSERFRAC5
105 / HUSERFRAC5
117 / HUSERFRAC5
128 / HUSERFRAC5
134 / HUSERFRAC5
137 / HUSERFRAC5
175 / HUSERFRAC5
241 / HUSERFRAC5
333 / HUSERFRAC5
355 / HUSERFRAC5
370 / HUSERFRAC5
397 / HUSERFRAC5
421 / HUSERFRAC5
552 / HUSERFRAC5
607 / HUSERFRAC5
890 / HUSERFRAC5
ALPHA 1 / HUSERFRAC5
UKN 12 / HUSERFRAC5
UKN 20 / HUSERFRAC5

Table 2: Non-Genetic Markers for Osteoporosis

MSN Number/Master
36 / HUSERFR3A
128 / HUSERFR3A
152 / HUSERFR3A
245 / HUSERFR3A
431 / HUSERFR3A
228 / HUSERFR6
243 / HUSERFR6
516 / HUSERFR6

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160 / HUSERFRAC5
183 / HUSERFRAC5
187 / HUSERFRAC5
244 / HUSERFRAC5
310 / HUSERFRAC5
607 / HUSERFRAC5
620 / HUSERFRAC5
856 / HUSERFRAC5
1042 / HUSERFRAC5
1249 / HUSERFRAC5
UKN 12 / HUSERFRAC5
UKN 6 / HUSERFRAC5

Table 3: Non-Genetic Markers for Diabetes

MSN Number/Master
14 / HUSERFR3A
28 / HUSERFR3A
332 / HUSERFR3A
832 / HUSERFR3A
1389 / HUSERFR3A
1865 / HUSERFR3A
S 15 / HUSERFR3A
S 27 / HUSERFR3A
101 / HUSERFRAC5

Table 4: Non-Genetic Markers for Osteoarthritis

MSN Number/Master

1992 / HUSERFR6

Table 5: Non-Genetic Markers for Hypertension

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MSN Number/Master

520 / HUSERFR3A
1862 / HUSERFR3A
S 20 / HUSERFR3A
468 / HUSERFR6
2184 / HUSERFR6
2335 / HUSERFR6
2391 / HUSERFR6
347 / HUSERFRAC5
800 / HUSERFRAC5

Table 6: Identification of the Protein Spots

MASTER	MSN	stain	trait	type	pi	mw
HUSERFR3A	14	AG	INSRES_z	correlation	6.22	139364
HUSERFR3A	19	CB	TFM_z	anova	4.12	70122
HUSERFR3A	28	AG	INSRES_z	anova	4.2	61684
HUSERFR3A	36	AG	SBMD_z	correlation	5.12	82460
HUSERFR3A	128	CB	TOTBMD_z	anova	5.57	74350
HUSERFR3A	152	CB	TOTBMD_z	anova	5.98	73753
HUSERFR3A	245	AG	SBMD_z	correlation	5.07	82737
HUSERFR3A	316	AG	PCTFAT	correlation	5.51	63087
HUSERFR3A	332	AG	INSRES_z	anova	5.13	81527
HUSERFR3A	420	CB	TFM_z	correlation	5.1	109205
HUSERFR3A	431	AG	TOTBMD_z	correlation	6.14	55277
HUSERFR3A	501	CB	TFM_z	anova	5.64	30450
HUSERFR3A	520	CB	RCAI	correlation	5.22	42485
HUSERFR3A	680	AG	TFM_z	correlation	4.99	116640
HUSERFR3A	832	AG	INSRES_z	correlation	5.33	44792
HUSERFR3A	1139	AG	TFM_z	correlation	4.95	117289
HUSERFR3A	1389	AG	INSRES_z	correlation	6.31	29942

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HUSERFR3A		1862	CB	RCAI	correlation	5.09	43239
HUSERFR3A		1865	AG	INSRES_z	correlation	4.48	64489
HUSERFR3A	S15		AG	INSRES_z	anova_pop_position		
HUSERFR3A	S2		CB	TFM_z	cor_pop_position		
HUSERFR3A	S20		CB	RCAI	cor_pop_average		
HUSERFR3A	S22		CB	TFM_z	cor_pop_position		
HUSERFR3A	S24		AG	TFM_z	cor_pop_position		
HUSERFR3A	S27		AG	INSRES_z	anova_pop_average		
HUSERFR3A	S33		CB	TFM_z	anova_pop_average		
HUSERFR6		228	AG	TOTBMD_z	correlation	5	113862
HUSERFR6		243	AG	TOTBMD_z	correlation	5.15	31818
HUSERFR6		468	AG	RCAI	correlation	4.98	39037
HUSERFR6		516	AG	TOTBMD_z	correlation	5.55	54703
HUSERFR6		519	AG	PCTFAT	correlation	4.89	113581
HUSERFR6		1992	AG	OVE_z	correlation	4.99	31420
HUSERFR6		2184	AG	CCAI_z	anova	5.21	85763
HUSERFR6		2335	AG	CCAI_z	anova	5.68	79223
HUSERFR6		2391	AG	RCAI	correlation	4.82	44734
HUSERFRAC5		39	CB	TFM_z	correlation	6.45	78358
HUSERFRAC5		72	CB	TFM_z	anova	4.94	42730
HUSERFRAC5		96	CB	TFM_z	correlation	6.19	79944
HUSERFRAC5		101	AG	INSRES_z	anova	5.59	47373
HUSERFRAC5		101	AG	TFM_z	correlation	5.59	47373
HUSERFRAC5		104	CB	TFM_z	correlation	6.27	58175
HUSERFRAC5		105	CB	TFM_z	correlation	5.35	37624
HUSERFRAC5		117	CB	TFM_z	correlation	6.82	60637
HUSERFRAC5		117	CB	PCTFAT	correlation	6.82	60637
HUSERFRAC5		128	CB	TFM_z	correlation	6.62	60420
HUSERFRAC5		134	AG	TFM_z	correlation	6.55	57014

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HUSERFRAC5	134 CB	TFM_z	correlation	6.55	57014
HUSERFRAC5	137 AG	TFM_z	correlation	5.5	47784
HUSERFRAC5	160 AG	SBMD_z	correlation	5.34	88883
HUSERFRAC5	175 CB	TFM_z	correlation	5.24	85833
HUSERFRAC5	183 AG	SBMD_z	correlation	5.38	88728
HUSERFRAC5	183 CB	SBMD_z	correlation	5.38	88728
HUSERFRAC5	187 CB	SBMD_z	correlation	5.87	67324
HUSERFRAC5	241 CB	TFM_z	anova	5.42	88879
HUSERFRAC5	244 CB	TOTBMD_z	anova	6.67	72180
HUSERFRAC5	310 CB	SBMD_z	correlation	5.45	89277
HUSERFRAC5	333 CB	TFM_z	correlation	6.1	79895
HUSERFRAC5	347 CB	RCAI	correlation	5.22	37586
HUSERFRAC5	355 AG	TFM_z	correlation	6.46	61569
HUSERFRAC5	370 CB	TFM_z	correlation	5	79599
HUSERFRAC5	397 CB	PCTFAT	correlation	5.35	29723
HUSERFRAC5	421 AG	TFM_z	correlation	5.81	80243
HUSERFRAC5	552 CB	TFM_z	anova	5.74	79735
HUSERFRAC5	607 CB	TFM_z	correlation	5.42	44992
HUSERFRAC5	607 CB	SBMD_z	correlation	5.42	44992
HUSERFRAC5	620 AG	SBMD_z	correlation	5.94	67780
HUSERFRAC5	800 AG	RCAI	correlation	5.23	28230
HUSERFRAC5	856 AG	SBMD_z	correlation	6.03	71694
HUSERFRAC5	890 AG	TFM_z	anova	6.89	32227
HUSERFRAC5	1042 AG	SBMD_z	correlation	6.24	70636
HUSERFRAC5	1249 CB	SBMD_z	correlation	5.81	66964
HUSERFRAC5	ALPHA1 AT CB	TFM_z	anova_pop_average		
HUSERFRAC5	UNK12 CB	SBMD_z	anova_pop_average		
HUSERFRAC5	UNK12 CB	TFM_z	cor_pop_average		
HUSERFRAC5	UNK20 AG	TFM_z	cor_pop_average		

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HUSERFRAC5UNK20	CB	TFM_z	cor_pop_average
HUSERFRAC5UNK21	CB	TFM_z	cor_pop_average
HUSERFRAC5UNK6	CB	SBMD_z	cor_pop_average
HUSERFRAC5UNK6	CB	SBMD_z	anova_pop_position

The pI and MW are predicted from the warped and impressed locations on the two dimensional gel. The precision is generally very good but may vary as much as approximately ± 0.5 pI units and ± 10% in molecular weight.

The ANOVA analysis sought consistent increase of decrease in protein abundance for the twin with the higher expression of the disease trait. This was a two-way ANOVA analysis based on the following simultaneous criteria: probability is <0.01, binary twin (lower twin value =0, higher twin value =1) is <0.01 and N (number of discordant twin pairs) >8 subjects. The overall correlation criteria is p<0.01 for Pearson correlation between trait and protein spot volume, p<0.01 for Spearman correlation between trait and protein spot volume, p<0.1 for Spearman correlation between trait and protein spot volume (discordant pair subjects), p<0.01 for Pearson correlation between twins, and p<0.01 for Spearman correlation between twins.

Even though the protein may not be heretofore isolated or characterized, the present invention effectively isolates and characterizes the proteins. From the MSN number given above, one has a unique isolated protein from a spot on the 2-dimensional electrophoresis gel. The relative molecular weight and relative pI for each spot are determinable by reference to established landmark proteins, which are fully characterized by sequencing, and a theoretical molecular weight and pI calculated. By plotting the theoretical values on a graph and

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comparing the location of the previously unknown spot, these identifying features are determined. See Anderson et al, Electrophoresis 16:1977-1981 (1995) for more details, the contents of which are specifically incorporated by reference. This provides a reproducible method for isolating the protein markers of the present invention.

To confirm the landmarks, biological samples from the present experiment were mixed with very well characterized biological samples before separation and quantification. By co-separating them, and comparing the results with the very well characterized biological samples' proteins, one may confirm identification of a common protein and/or extrapolate pI and molecular weight values for each spot.

The Figures 1-10 show the placement of each spot relative to other spots in the two-dimensional electrophoresis gel.

While it is very useful to know the quantities of various protein ligands in a sample, in some situations, it may be useful to compare the sample to a standard or to measure differences in concentrations of various ligands from another sample. For example, disease specific makers may be deduced by determining which proteins are in higher or lower concentrations in a sample from a diseased individual as compared to a normal individual. The differential may be determined by using the present invention to determine the quantities in a normal and a diseased sample. The results from each experiment are compared to generate the differential results.

A particular protein level may be compared to total protein levels in the sample if a concentration control is desired. This will generate a coefficient to compare to standards so that control need not be run side by side every time. Total protein may be determined by measuring total protein being loaded on the

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gel, but preferably, it is compared to all other spots in the 2DE gel or even total protein in a sample. Alternatively, one may compare a particular protein to a standard protein in the sample (natural internal control) or added to the sample (added internal control).

Proteomic techniques were used to study proteome changes in biological samples from diseased and normal genetically identical twins. The diseases were found to induce a complex pattern or "signature" of alterations in biological sample proteins, some of which are probably related to the disease process and others simply unrelated markers altered by the individual's response to the disease state.

Numerous changes in the proteome of serum from individuals with obesity, osteoporosis, diabetes, osteoarthritis and hypertension were observed. For obesity, total fat mass and percentage of fat were measured. For diabetes, Insulin resistance, fasting insulin levels and central fat mass were measured. For osteoporosis, bond density, SBMD and TOTBMD were measured. For osteoarthritis, joint space narrowing as detected by X-ray or OVE were measured. For hypertension/arterial distensibility, the following were measured arterial tonometry, pulsewave velocity, CCAI and RCAI.

When determining what is to be considered a protein marker, combinations of proteins may constitute a combination marker of disease state or efficacy of treatment. Even when two or more proteins are not sufficiently statistically significant to be considered markers by themselves, when considered in combination, the combination marker may be statistically significant. This is done by determining proteins that are at altered abundances in biological samples from diseased states compared to normal controls. Selecting two proteins that are

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less than statistically significant markers by themselves, one may combine the values in various ways for two or more of these proteins and determine whether the combination of values is altered in a statistically significant manner. Combination markers result when statistically significant differences between biological samples from diseased individuals and biological samples from control individuals are determined. Suitable data mining reveals a number of combination markers, and the theoretical rationale for some of these combination markers is still being determined.

Testing samples from subjects treated with therapeutics having different mechanisms of action is particularly preferred when searching for new candidate drugs of potentially new mechanisms of action. Markers common with different therapeutics represent a secondary pharmaceutical function. By comparing protein marker effects when using different pharmaceuticals, less than statistically significantly changed proteins may become protein markers.

An index marker is similar to a combination marker except that each protein in the index is itself already statistically significant as a protein marker alone. An index marker is an aggregate of plural significant protein markers which taken together and compared to the same index marker of a different sample. The index marker is then an extremely significant combination. For example, using a combination of markers, each with $p<0.001$, may yield an index marker of $p<0.00001$ or lower.

Protein markers may be found altered by the same general disease by different causes represent different categories. Producing the same markers are perhaps the best markers for screening new candidate drugs for a given indication because they are not mechanism of action specific. These are believed

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to reveal elements common to the mechanisms of action of the different pharmacological classes on a particular disease state. Such a marker is good for screening for drugs having completely unknown modes of action but directed to a similar disease treatment objective.

By using a different method for measuring the proteome, different markers may also be uncovered. Presently two-dimensional electrophoresis is the preferred method for measuring the proteins in a proteome. However other techniques such a plurality of different chromatography methods may be used. Even within a preferred method for measuring proteins in a proteome, different variations reveal different proteins. For example, different protein solubilizing solutions and different gradients affect which proteins will be observed on a two-dimensional electrophoresis gel. Furthermore, by comparing how one protein changes in abundance with respect to others, still other protein markers may be found.

This method is performed by comparing all proteins that change in abundance in the same or opposite direction as known protein markers. Even if the change in abundance of the proposed protein marker is not changed significantly, the fact that its abundance changes along with established protein markers indicates it may be an acceptable marker.

Another method for finding a marker even when the data is not statistically significant is to determine whether a protein is altered in tandem with known protein markers. Proteins that are not sufficiently altered to be considered protein markers are called protein "submarkers" when they have altered levels in tandem or opposite direction and magnitude when consistent among a group of samples. The direction and amount of alteration between the control and disease samples is noted. This is

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compared across multiple individuals and compared to established protein markers. Tandem moving protein submarkers that are altered both in direction and in amount between individuals and paralleling known protein markers may then be considered to be "protein markers" in their own right. Such may then be assayed for the multitude of purposes as any other marker.

Another method for measuring the proteins in a two-dimensional electrophoresis gel is by determining qualitatively whether a protein is present or absent. For example, a protein found in a biological sample from a control but not in a comparable sample from a disease sample would be of particular interest as it represents that the disease state completely eliminated the protein marker. Likewise, the reverse where a protein is induced only in diseased state but not controls is also of particular interest. A p value is not even calculable in these situations as one is comparing to zero.

Another qualitative or quantitative change in protein marker levels is in the presence of or amount of protein variants and the ratios between them. Some disease states are known to alter glycosylation and any candidate compound being tested may induce a different abundance of protein variants. Likewise, cleavage fragments (or the lack thereof) may be in altered abundance. Still further, enzymes may be in the same concentration but have dramatically different activity due to various agents such as cofactors, metal ions, vitamins etc. In all of these situations, the altered level or change in abundance of a protein or its variant(s) may be used to serve as a suitable marker for disease status. This may be observed as a shift in spot location or new spot formation.

Some diseases actually have different causes and may result in different markers. For example, a headache may be caused by

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literally dozens of different problems. To best determine which apparently the same disease have an unrelated mechanism, it is desirable to compare to a composite effect of many drugs and other therapeutic agents, preferably from a large proteomics database. The comparison to the positive control same mechanism of action and the negative control same mechanism of action may be seen as agonist/antagonist effects and correlations between these two control groups provides a further source for protein markers.

Diagnostic uses for the markers are not limited to measuring the proteome for each biological sample. Once one or several critical markers are determined, these proteins alone may be assayed for as a way to test for diagnosing the disease state, its prognosis, treatment choices and monitoring response. A number of protein assays are known per se and they vary depending on the protein being measured. Of particular interest are immunoassays as they are fast, inexpensive and relatively simple to perform.

The preparation of antibodies to known isolated proteins is well known per se. In the present invention, it may be useful to prepare both monospecific antisera, which ideally will be affinity purified before use, and monoclonal antibodies. For diagnostic purposes, monoclonal antibodies are usually preferred to enhance uniformity and specificity. For immunosubtraction procedures, antisera is usually preferred as total antigen binding is what is most critical and multiple antibody clones provide enhanced binding. Other specific ligands may be used such as recombinant antibodies, single chain antibodies, antibody display phage, selected members from combinatorial libraries and the like.

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Diagnostic reagents and kits of the present invention are typically used in a "sandwich" format to detect the presence or quantity of proteins in a biological sample. A description of various immunoassay techniques is found in BASIC AND CLINICAL IMMUNOLOGY (4th ed. 1982 and more recent editions) by D. P. Sites at al., published by Lange Medical Publications of Los Altos, Calif., and in a large number of U.S. Patents including 3,654,090, 3,850,752 and 4,016,043, the respective contents of which are incorporated herein by reference.

In a preferred embodiment, the kit further includes, a labeled component that is bound to or is bindable to the detection reagents or the protein being assayed or both. Also, in a separate package, an amplifying reagent such as complement, such as guinea pig complement, anti-immunoglobulin antibodies or S. aureus cowan strain protein A that reacts with the antigen or antibodies being detected. In these embodiments, the label specific binding agent is capable of specifically binding the amplifying means when the amplifying means is bound to the protein or antibody.

Important to the labeling and detection systems is the ability to determine quantity of label present to quantify the ligands present in the original sample. Since the signal and its intensity is a measure of the number of molecules bound from the sample and hence of the number of receptors bound, the number of ligand molecules in the original sample may be determined. Optical and electrical signals are readily quantifiable. Radioactive signals may also be quantifiable directly but preferably is determined optically by use of a standard scintillation cocktail.

While the receptors most commonly utilized are antibody molecules, or a portion thereof, one may equally use other

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specific binding receptors such as hormone receptors, intracellular signal receptors, certain cell surface proteins (also called RECEPTORS in the scientific literature), an assortment of enzymes, signal transduction and binding proteins found in biological systems.

Likewise, ligands exemplified as proteins below may also be small organic molecules such as metabolic products in a cell. By simultaneously detecting many or all metabolites in a sample, one can determine the global effects of an effector on the cell. Effectors may be the disease state itself, drugs, toxins, infectious agents, physiological stress, environmental changes, etc.

As the number of markers found is large, a simultaneous multiple assaying systems such as a microarray of binding agents for each desired protein marker is preferred. In such a microarray, a specific binding receptor for each protein marker ligand, e.g. an antibody, is immobilized at a different address and contained in a distinct region of the microarray or bound to a distinct particle or label. The protein marker ligand containing sample is then contacted to the microarray and allowed to bind. Binding may then be detected by a number of techniques, known per se, particularly preferred being binding a labeled receptor to one or more components of a ligand/receptor complex and detecting the label.

Microarrays containing multiple receptors are known per se. An earlier discovery of a test strip with multiple receptors has been commercially used for decades. A number of designs for multiple simultaneous binding assays are known per se in the analytic testing field.

The array may utilize antibody or other receptor display phage as a binding agent or an immobilizing agent for the

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protein marker ligand. Either the receptor alone or the whole display phage may be used. When used as an immobilizing agent, different cells of the microarray contain a different receptor. When used as a labeled binding agent, the receptor or phage may be labeled (before or after binding to the ligand) by a number of techniques (such as direct fluorescent dyes, e.g. TOTO-1, labeled protein A or G, labeled anti-Ig, etc.) and utilized without prior identification of which display phage contains a particular antibody as an initial immobilized capture receptor performs the discrimination.

Other competitive techniques using a microarray of immobilized protein markers and labeled or labelable receptors may also be used.

The techniques described in provisional patent application 60/166,266 filed November 18, 1999 of N. Leigh Anderson may be employed to measure a very large number of proteins simultaneously, including any or all of those in a pathway relating to a disease state. Such a technique may be applied to detecting any or all of the protein markers of the present invention.

For microarrays that are not a unitary solid phase, multiple different beads, each with a different label or having a different combination of labels may be used. For example a bead having different shades of a chromagen or different proportions of different chromagens or other detectable features. Each bead or set of beads with the same identifying label(s) is to have an immobilized ligand or receptor. Individual sets of beads may be identified in a mixture by spreading on a flat surface and scanning or by moving the beads past a detector. The combination of the labels and the bead label(s) provides identification of the ligand of interest in

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the sample. The numerical ratio of beads having labels to beads without labels or with different labels provides a quantitative measurement. Just as the sample may be deduced from which addresses contained labels in a traditional microarray, with plural unique beads, the address may be deduced by determining which bead contains their corresponding label(s).

Once one has the isolated protein on a two-dimensional electrophoresis gel or in other isolated form, the protein may be identified. If the protein is known and the gene cloned, one can then produce large quantities of protein by conventional recombinant DNA methods. Likewise, if the protein is not known or is known but the gene not cloned, the amino acid sequence may then be determined by sequencing, mass spectrometry or other methods well known per se. One may deduce the possible nucleotide sequences from the amino acid sequence and use such probes to isolate the gene using well-known techniques known per se to obtain the gene.

As an alternative to, or preferable in conjunction with, measuring the amount of a protein marker of interest in a biological sample, one may also measure the level of mRNA for the protein marker. This level of mRNA may be measured in absolute levels or relative to all other or specific other mRNA. One may even correlate between protein concentrations and mRNA concentrations if so desired.

For therapeutic purposes, pharmaceutical compositions in the form of small organic molecules, peptides, proteins, antibodies or other specific binding receptors, which may act as agonists or antagonists for the protein markers, may be used. The protein constituting the marker itself may be a functional active ingredient as well. Compositions that regulate expression of the gene encoding the marker, such an antisense

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may also be used. Each of these classes of pharmaceuticals has been used previously against other drug discovery targets and is thus likely results from the drug discover targets offered by the present invention.

Pharmaceutical compositions may be prepared for use in humans or animals via the oral, parenteral, aerosol or rectal route, in the form of wafer capsules, tablets, gelatin capsules, powders,
drinkable solutions, injectable solutions, including delayed forms and sustained-release dressings for transdermal administration of the active principle, nasal sprays, or topical formulations (cream, emulsion, etc.), comprising a compound interacting with a marker of the present invention and at least one pharmaceutically acceptable carrier. The pharmaceutical compositions according to the invention are advantageously dosed to deliver the active principle in a single unit dose.

For oral administration, the effective unit doses are between 0.1 μg and 500 mg. For intravenous administration, the effective unit doses are between 0.1 μg and 100 mg.
According to the invention, the pharmaceuticals are preferably administered orally, for example, in the form of tablets, dragees, capsules, solutions, or intraperitoneally, intramuscularly, subcutaneously, intraarticularly or intravenously, for example, by means of injection or infusion.
It is especially preferred that the application according to the invention occurs in such a manner that the active agent is released with delay, that is as a depot.
Unit doses can be administered, for example, 1 to 4 times daily. The exact dose depends on the method of administration and the condition to be treated. Naturally, it can be necessary to vary

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the dose routinely depending on the age and the weight of the patient and the severity of the condition to be treated.

While the present invention is discussed in terms of the protein markers, their methods for preparation and uses for diagnostic, therapeutic and drug discovery; the markers may be produced by other known methods and used for other known uses for proteins. For example, once the marker has been identified, it may be produced by extraction from a biological sample or the gene cloned and expressed to produce the protein. Such methodology is well known in the art. Likewise, protein markers have been used for a number of basic research and identification uses such as in pathology, forensics and archeology.

EXAMPLE 1: SAMPLE SELECTION

Approximately 400 pairs of monozygotic human twins were screened for divergent phenotypic disease states. Serum samples from 158 subjects (79 twin pairs) were selected based on differences in five disease states. The samples were divided into 5 discordant disease groups according to intra-twin clinical trait differences. The quantitative traits were measured to determine the clinical disease area given in the chart below.

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Group Number	Clinical Disease Area	Quantitative Trait
1	Obesity	Total Fat Mass, Percentage of Fat
2	Diabetes	Insulin Resistance
3	Osteoporosis	SBMD/TOTBMD
4	Osteoarthritis	OVE
5	Hypertension/ arterial distensibility	CCAI/RCAI

The samples are whole serum with approximately 70 mg/ml proteins. The lipids did not significantly interfere with the chromatographic separation. For disease groups 1 and 2, 25 μl of total serum was used. For groups 3, 4 and 5, 50 μl was used.

EXAMPLE 2: SERUM FRACTIONATION

Protein subtraction columns were prepared to remove common proteins that comprise most of the protein in the sample. For groups 1, 2 and 3, two subtraction columns were prepared and used. The first column (ATH) contained Poros® beads covalently bound to Protein A, Protein G or a mixture of the two, which is then bound to monospecific antisera to certain serum proteins. The antibodies were specific to albumin, transferrin and haptoglobin. The second column contained immobilized wheat germ agglutinin lectin. For groups 4 and 5, the first column had antibody to alpha-1 antitrypsin, albumin, transferrin and haptoglobin with a second column of immobilized Protein A. All antibodies were crosslinked according to the method of Schneider et al, Journal of Biological Chemistry 257:10766-10769 (1982).

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Approximately 4 ml of immunoaffinity resin was used. Generally, the columns completely removed all of the components, which they specifically bound except for group 3 where a small amount of albumin was carried over.

Samples of about 70 mg/ml protein were used with 25-50 µl being added which corresponds to about 1.7 mg to 3.4 mg. The samples were loaded into the first HPLC column. Unbound protein fraction from the ATH column was eluted by eluted in 0.5 M ammonium bicarbonate and transferred to the second column. A first unbound protein fraction was eluted with 0.5 M ammonium bicarbonate buffer followed by eluting a bound second protein fraction with 0.5 M N-acetyglucosamine followed by 0.5M ammonium bicarbonate to wash the protein through. UV280nm detected peak areas were observed continually as controls for reproducibility of serum loading and column performance.

In this way, two fractions from each serum protein subtraction experiment were retained. The fractions containing proteins (albumins etc.) removed by the ATH column by HCL, pH 2.5 or acetic acid, pH 2.5-3 were discarded and the columns equilibrated to be reused for the next sample. Quantitatively, about half of the serum protein was removed from the sample. The first protein fraction contains non-glycosylated proteins without sialic acid chains and the second protein fraction contains glycosylated proteins. The final protein concentration ranged between 12 and 22 mg/ml in 0.5 M ammonium bicarbonate buffer.

Both collected fractions were collected in 2-4 ml 0.5 M ammonium bicarbonate buffer and underwent concentration to 100 µl followed by buffer exchange to 4 ml twice in 25 mMol ammonium bicarbonate by ultrafiltration on a membrane unit with an approximately 5,000 dalton molecular weight cut off. About 100

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μl is retained and lyophilized. The presence of proteins is visible because both fractions contain molecules (heme, iron, porphyrin, etc.) that absorb light in the visible range. This suggests that little loss of protein occurred during the process.

The fractions were resolubilized in 25-50 μl solubilizing solution below. About 2 mg protein is present (70 mg/ml plus solubilizing solution), thus some proteins were not solubilized under the denaturing conditions. 5-20 μl/gel is loaded for each sample.

For Groups 4 and 5, the methods above were repeated with the second column being immobilized protein A. The unbound proteins were recovered. An elution buffer of acetic acid, pH 3-2.5, equilibrated the column.

EXAMPLE 3: 2-DIMENSIONAL ELECTROPHORESIS

Protein aliquots (about 8 μl) of fractionated serum proteins were loaded onto the gels.

The samples were solubilized in 9M urea, 2% CHAPS, 0.5% dithiothreitol (DTT) and 2% carrier ampholytes pH 8-10.5.

Ultrapure reagents for polyacrylamide gel preparation were obtained from Bio-Rad (Richmond, CA). Ampholytes, pH 4-8, were from BDH (Poole, UK), ampholytes pH 8-10.5 were from Pharmacia (Uppsala, Sweden) and IGEPAL-630 was obtained from Sigma (St. Louis, MO). Deionized water from a high purity water system (Neu-Ion, Inc., Baltimore, MD) was used. System filters are changed monthly to ensure 18MΩ purity. Dithiothreitol (DTT) was obtained from Gallard-Schlesinger Industries, Inc. (Carle Place, NY). All chemicals (unless specified) were reagent grade and used without further purification.

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Sample proteins were resolved with two-dimensional gel electrophoresis using an automated and controlled versions of the 20 x 25 cm ISO-DALT® 2-D system (Anderson, 1991). Solubilized samples were applied to each IEF gel, and the gels were run for 25,550 volt-hours using a progressively increasing voltage with a high-voltage programmable power supply. An Angelique™ computer-controlled gradient-casting system (Large Scale Biology Corporation, Rockville, MD) was used to prepare the second-dimension SDS slab gels. The top 5% of each gel was 11%T acrylamide and the lower 95% of the gel varied linearly from 11% to 19%T for groups 1, 2 and 3. For groups 4 and 5, the top 5% of each gel was 8%T and the lower 95% the gel varied linearly from 8-15%T. The IEF gels were loaded directly onto the slab gels using an equilibration buffer with a blue tracking dye and were held in place with a 1% agarose overlay. Second-dimensional slab gels were either run overnight at 160 V in cooled DALT tanks (10°C) with buffer circulation and were taken out when the tracking dye reached the bottom of the gel for groups 1-3. For groups 4 and 5, the conditions were 2-3 hours at 600V and 20°C.

For Coomassie blue staining followed by silver staining for gels for groups 1-3, following SDS electrophoresis, the slab gels were fixed overnight in 1.5 liters/10 gels of 50% ethanol/3% phosphoric acid and then washed three times for 30 min in 1.5 liters/10 gels of cold DI water. They were transferred to 1.5 liters/10 gels of 34% methanol/17% ammonium sulfate/3% phosphoric acid for one hour, and after the addition of one gram powdered Coomassie Blue G-250 the gels were stained for three days to achieve equilibrium intensity.

Stained slab gels were scanned and digitized in red light at 133 micron resolution, using an Eikonix 1412 scanner and

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images were processed using the Kepler® software system as described (Anderson '94). Coomassie blue gels were destained in 1.5 L of 50% ethanol, 45% deionized water and 5% acetic acid overnight and reswell in DI water for one hour.

The gels were then clipped onto a gel hanger and processed through the fully automatic Argentron™ silver stainer. The individual steps include agitation for 30 seconds in deionized water, one minute in 0.44 g sodium thiosulfate in 2 L DI water, 10 seconds in deionized water, 30 minutes in 4.6 g silver nitrate in 2L DI water and 0.78 ml 37% formaldehyde, 10 second DI water wash, 20 minutes in 66 g potassium carbonate, 0.033 g potassium thiosulfate in 2L deionized water with 0.78 ml of 37% formaldehyde. Images are taken at 30 second intervals and the development is stopped 88 g tris (hydroxymethyl)aminomethane in 2 L deionized water and 44 ml glacial acetic acid.

For groups 4 and 5, the gels were fixed in 1.5 L of 50% ethanol and 3% phosphoric acid in 47% deionized water for 4 hours and then washed in DI water for 1 hour. The gels are clipped into gel hanger and processed as above.

The images were assembled and then processed using the Kepler® software system as described above for the silver stained gels.

EXAMPLE 4: DETERMINATION OF PROTEIN MARKERS

The Coomassie blue stained gels averaged a few hundred quantifiable protein spots per gel while silver stained gels averaged between one and one-half and twice as many spots per gel. The samples were mixed with rat liver homogenate, a well-characterized sample where a very large number of proteins have been completely identified. From the co-electrophoresis gel the sample's spots were "WARPED" using the method of U.S. Serial

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Number 643,675 filed August 24, 2000, and the "IMPRESS" method of U.S. Serial Number _____ filed August 31, 2000, Attorney Docket Number 40732. The data regarding the protein spots identified is given in the Tables above and Figures.

It will be understood that various modifications may be made to the embodiments disclosed herein. Therefore, the above description should not be construed as limiting, but merely as exemplifications of preferred embodiments. Those skilled in the art will envision other modifications within the scope and spirit of the claims appended hereto.

All patents and references cited herein are explicitly incorporated by reference in their entirety.

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